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Construction of a human Semaphorin 5 expression plasmid

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CONSTRUCTION OF A HUMAN *SEMAPHORIN 5* EXPRESSION PLASMID

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Edwin Haghnazari

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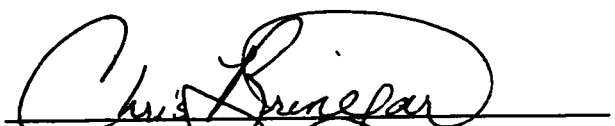
APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES

Dr. David Matthes



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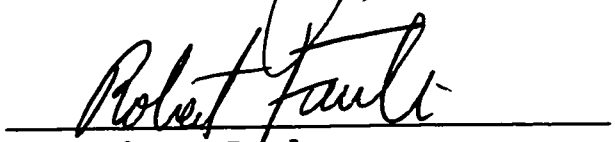

Dr. Robert Fowler

APPROVED FOR THE UNIVERSITY

ABSTRACT

CONSTRUCTION OF A HUMAN *SEMAPHORIN 5* EXPRESSION PLASMID

by Edwin Haghnazari

Chromosomal region 3p21.3 is deleted in nearly 100% of small cell lung cancer (SCLC) cells examined, suggesting the presence of one or more tumor suppressor genes in this region. Human *Semaphorins 4* and *5* are located within 3p21.3. Northern blot analysis has revealed that only 4% of SCLC cell lines express the gene for Semaphorin 5, making this protein a strong candidate.

This thesis has set the foundation for a detailed study of tumor suppression by human Semaphorin 5 in NCI-H209 cells. The gene for this potential tumor suppressor has been subcloned into the mammalian expression vector pCDNA3.1/zeo™ and fused in-frame with a myc epitope tag to allow detection. The construct has been sequenced and found to be at least 99.9% identical with the published data. Additionally, a zeocin™ concentration of 250 µg/ml has been determined to be sufficient to eliminate ~90% of untransfected NCI-H209 cells 6 days after transfection.

ACKNOWLEDGMENTS

I would like to first of all extend my greatest thanks and appreciation to Dr. David Matthes for accepting me as his graduate student and helping me throughout my project. I would also like to thank Dr. John Minna (UT Southwestern Medical Center) for providing me with the necessary cDNA clone. Dr. Harris Goonewardena and Winston Wu have been extremely helpful with their great technical and theoretical expertise. I am also grateful for the continuous support of Drs. Chris Brinegar, Steven White, Ruth Kibler, and Robert Fowler.

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Chapter 1

Introduction

[1.1] Small Cell Lung Carcinoma

Small cell lung cancer (SCLC) is a highly malignant form of bronchogenic carcinoma. It occurs mainly in middle aged individuals with a history of tobacco smoking. SCLC accounts for about 25% of the 110,000 new cases of lung cancer that occur yearly in the US (Whang-Peng et al. 1982a). This form of lung cancer is clinically distinct from other types of lung cancer; it is characterized by small, oval shaped cells and is usually highly metastatic to the hilum of the lung (the depression where nerves and blood vessels enter) and lymph nodes. Therefore, unlike other forms of lung cancer, surgery is not performed. The recommended forms of therapy are chemotherapy and radiation (Whang-Peng et al. 1982a).

Loss of DNA from the short arm of human chromosome 3 (3p) is a critical event in the pathogenesis of a number of cancers, including lung (Brauch et al. 1987; Naylor et al. 1987; Kok et al. 1987; Popescu, Chahinian, and DiPaolo 1988), renal (Zbar et al. 1987), breast (Ali, Lidereau, and Callahan 1989), ovarian (Devilee et al. 1989), testicular

(Lothe et al. 1989), and head and neck (Naggar et al. 1993). Several studies have indicated that putative lung cancer suppressor genes reside on chromosome 3p (Whang-Peng et al. 1982a; 1982b; 1991; Naylor et al. 1987; Hibi et al. 1992; Kok et al. 1994). Deletions of chromosome 3p in SCLC have been observed in nearly all cases examined karyotypically (Whang-Peng et al. 1982a).

Studies have shown 3p loss of heterozygosity in >90% of SCLCs examined and indicate the presence of tumor suppressor genes located in one or more of the following regions: 3p12, 3p13, 3p21.3, and 3p25 (Brauch et al. 1987; Hibi et al. 1992; Sekido et al. 1996). The 3p21.3 region is particularly interesting because it is deleted in nearly 100% of SCLC cases (Hibi et al. 1992). Other experiments have shown that a fragment of chromosome 3 encompassing part of this 3p21.3 region can suppress tumor formation by mouse fibrosarcoma cells in athymic nude mice (Killary et al. 1992). This evidence strongly suggests the presence of one or more tumor suppressor gene(s) within the chromosome 3p21.3 region.

[1.2] Semaphorins

Semaphorins (originally defined by proteins Fasciclin IV and Collapsin) were initially discovered in grasshopper embryos as molecules involved in axonal growth cone guidance during development (Kolodkin et al. 1992). *Fasciclin IV* was shown to be expressed on axon pathways in the developing CNS as well as on circumferencial bands of the epithelium in the developing limb buds. It was shown that the growth cones, during their migration, made characteristic turns once they encountered the bands where *Fasciclin IV* was expressed. It was also shown that the use of antibodies against this molecule caused aberrant axonal growth; in antibody treated limb buds, the nerve axons exhibited abnormal morphology beginning at the site of *Fasciclin IV* expression. Subsequent cloning revealed that Fasciclin IV is a novel integral glycoprotein with a distinctive pattern of cysteines in its extracellular domain. Fasciclin IV was later renamed grasshopper Semaphorin I.

There are five types of Semaphorins (Table 1): transmembrane (type I); secreted with an Immunoglobulin (Ig) domain (type II); secreted with an Ig domain and a charged C-terminus (type III); transmembrane with an Ig

domain (type IV); and transmembrane with Thrombospondin repeats (type V) (Adams, Betz, and Puschel 1996). All five types have a conserved extracellular "Semaphorin domain" of approximately 500 amino acids with 16 cysteine residues (Kolodkin, Matthes, and Goodman 1993).

Table 1
The five types of Semaphorins with their unique domain compositions

Type I	Transmembrane
Type II	Secreted with an Ig domain
Type III	Secreted with an Ig domain and a charged C-terminus
Type IV	Transmembrane with an Ig domain
Type V	Transmembrane with thrombospondin repeats

Subsequent analyses revealed that *Semaphorins* are expressed by a variety of non-neuronal tissues. It was shown that *Drosophila Semaphorin II*, for example, in addition to being expressed in the nervous system, is also expressed in thoracic muscle fibers and the gonads. Its loss leads to greatly reduced eclosion (emergence of adult fly from pupal state), flightlessness, behavioral defects, death two days after eclosion, as well as an inability to drink (Kolodkin, Matthes, and Goodman 1993).

In humans, *Semaphorins 4* and *5* are expressed by a variety of tissue such as prostate, testes, ovaries, and brain (Sekido et al. 1996). The functions of these two proteins are currently unknown. Human CD 100, the first Semaphorin shown to function in the immune system, was found to induce B cell aggregation and improve B cell viability *in vitro* (Hall et al. 1996). This was further confirmation that Semaphorins play a role in the non-neuronal tissue.

Expressed sequence tag analysis suggests that there are as many as 20 human Semaphorins (Adams, Betz, and Puschel 1996). Two of the cloned *Semaphorins, 4* and *5,* have been shown to reside on chromosome 3p21.3, a region deleted in SCLC (Sekido et al. 1996). These two *Semaphorin* genes were shown to be part of a ~350 kbp common deletion region of all SCLC cells examined. They are approximately 70 kbp apart with two GTP binding protein genes, *GNAI-2* and *GNAT-1,* located in between them. Semaphorins 4 and 5 do not have a transmembrane domain, suggesting that they are both secreted proteins. Both of these Semaphorins are of type III because of the presence of an Ig domain and a charged C-terminus (Sekido et al. 1996; Xiang et al. 1996). Furthermore, Semaphorin 5 has been detected only in the

endoplasmic reticulum, supporting the prediction that this protein is secreted (Sekido et al. 1996).

Four other genes have been identified in the 3p21.3 region; *Dystroglycan* (*DAG1*), which codes for a laminin receptor that links the extracellular matrix and sarcolemma in skeletal muscle, *Acylpeptide hydrolase* (*APEH*), and two genes encoding zinc finger proteins, ZnF3 and ZnF16, which are most likely transcription regulators functioning in growth and differentiation (Roche et al. 1996). None of these four genes are contained in the smallest common deletion region (~350 kbp) of the SCLC cell lines examined, decreasing the likelihood of any of these proteins being tumor suppressors (Roche et al. 1996; Sekido et al. 1996).

Semaphorins 4 and *5* show distinct expression patterns among SCLC cells. Northern blot analysis of total RNA has revealed that only 4% (1/23) of SCLC cell lines express *Semaphorin 5*. The expression of *Semaphorin 4*, however, is quite different; northern blot analysis showed expression in 83% (19/23) of SCLC cell lines. The fact that only 4% of SCLC cells expressed the gene for Semaphorin 5, strongly suggests the possibility of this protein being a tumor suppressor.

If Semaphorins repel growth cones, they might also be involved in cell-cell interactions, such as contact inhibition, in non-neuronal tissue. Loss of contact inhibition in cancer cells could occur because of alterations or deletions of *Semaphorin* expression and/or function.

[1.3] Tumor Suppressor Genes

Tumor suppressor genes (also known as anti-oncogenes) are a class of genes believed to be involved in normal aspects of control of cellular growth and division. Their common characteristic is that their inactivation, usually by genetic means, contributes to tumor development. This contrasts with the other main class of genes involved in neoplasia, the dominantly acting oncogenes whose *activation*, again by various genetic means, also leads to malignant phenotype.

As the normal functioning of these genes suppresses tumorigenicity, regions of chromosomes that are consistently lost in tumors are likely to carry these tumor suppressor genes. Karyotype analysis of a wide variety of tumor types has shown that chromosomal deletions occur with a high frequency and in a tumor-specific manner, and

therefore, point to the likely location of these genes. Restriction fragment length polymorphism (RFLP) analysis, which allows comparison of a patient's normal and tumor genotype, has confirmed the occurrence of allelic loss inferred from cytogenetic analysis and has enabled investigators to search for these anti-oncogenes.

RFLP analysis has led to localization and isolation of several tumor suppressor genes. The most extensively studied tumor suppressor genes are *RB1* and *p53*. Both code for nuclear phosphoproteins, which are involved in cell cycle regulation (Lane and Crawford 1979; Lee et al. 1988). The transforming proteins of several DNA tumor viruses can form complexes with RB protein and the protein product of the *p53* gene. It has been shown that this interaction is necessary for the transformation process (Green 1989). The product of the *WT1* gene, a gene isolated from a region occasionally deleted in Wilms' tumor, is a protein with four zinc fingers, and therefore, likely to be transcription factor (Call et al. 1990; Gessler et al. 1990). The *NF1* gene product, thought to be involved in the development of neurofibromatosis, appears to be a GTPase activating protein, which interacts with p21 *ras* and may thereby regulate cell proliferation through signal

transduction pathways that involve p21 *ras* (Martin et al. 1990; Xu et al. 1990). The protein product of the *DCC* gene (deleted in colon carcinoma) is a cell surface molecule with considerable homology to neuronal cell adhesion molecules such as NCAM, that presumably regulate cell behavior through interactions with other cells (Fearon et al. 1990; Marshall 1991). Its reduced expression in tumors, as well as point mutations, insertions, and deletions within the gene in colon tumors, provide strong evidence that *DCC* is a tumor suppressor (Marshall 1991).

[1.4] Initial specific aims

The focus of this master's thesis project was to generate a mammalian expression construct of human *Semaphorin 5*, introduce the gene of this protein into a classic SCLC cell line (NCI-H209), and test for suppression of tumorigenicity using the following 5 assays:

[1.4.1] Contact inhibition assay

Unlike normal cells, tumor cells are not contact inhibited. In normal cells, cell division and cell motility are inhibited once cells contact each other. Tumor cells, however, continue to divide and proliferate

even when in close contact with neighboring cells. When in culture, NCI-H209 cells grow as densely packed floating aggregates, amorphous and irregular in outline, and lacking central necrosis (Carney et al. 1985). This assay will determine whether Semaphorin 5 is capable of causing contact inhibition among these SCLC cells.

[1.4.2] Soft agar clonogenic assay

Generally this assay is used to determine if a cancer cell line forms clumps (tumors) when unanchored to the bottom of a plate (Bookstein et al. 1990; Takahashi et al. 1991; Luo et al. 1995; Chen et al. 1996; Jin et al. 1996). Tumor cells have anchorage independent growth property; they will grow and form colonies (foci) on soft agar. Normal cells will not form any colonies on soft agar because they need to be anchored; hence they are anchorage dependent. This assay will determine if Semaphorin 5 is capable of restoring the anchorage dependent growth property of NCI-H209 cells.

[1.4.3] Radioactive thymidine incorporation assay

Since tumor cells are actively dividing cells, their rate of DNA synthesis will be greater than that of normal

cells. Thymidine is one of the bases that is incorporated into the newly synthesized DNA. This assay will measure the amount of incorporated radioactive thymidine, which should be proportional to DNA synthesis, and hence, cell division. This assay will determine if Semaphorin 5 is able to reduce the proliferation rate of NCI-H209 cells.

[1.4.4] Telomerase activity assay

Telomerase is the enzyme that adds hexameric nucleotide repeats onto ends of vertebrate chromosomal DNA (i.e. telomeres) to compensate for losses that occur with each round of DNA replication (Greider and Blackburn 1985). Terminal restriction fragments analysis has shown that chromosomes lose up to 200 nucleotides of their telomeric sequence for every cell division. None of the immortal (i.e. tumor) cells examined to date have shown any shortening or loss of their telomere sequences, suggesting that these telomeres are required for indefinite proliferation and malignancy (Counter et al. 1992; 1994a; 1994b). Telomerase enzyme activity has been shown to be consistently higher than normal in a variety of cancer cell lines, including SCLCs, and specifically NCI-H209 (Kim et

al. 1994). This assay will determine whether Semaphorin 5 is capable of reducing the activity of telomerase enzyme.

[1.4.5] In vivo tumorigenicity in nude mice

When tumor cells are injected into nude mice, which are immunologically compromised, tumors usually develop at the site of injection (Chen et al. 1996). NCI-H209 cells have been shown to cause tumor formation when injected into nude mice (Carney et al. 1985). This assay will determine if Semaphorin 5 is capable of inhibiting the tumorigenicity of NCI-H209 cells when injected into immunologically compromised mice.

[1.5] Actual work performed

Human *Semaphorin 5* was subcloned into the mammalian expression vector pCDNA3.1/zeo™ (Invitrogen, San Diego, CA). A small tag, the myc epitope, was inserted in-frame at the carboxy terminus to allow for detection of the protein by using anti-myc antibodies (there are currently no antibodies available for human Semaphorin 5). Both strands of the pCDNA3.1-HS5-myc construct were sequenced. The sequence data was compared to the published sequence (GenBank U28369) and found to be identical with the

exception of two bases, for which there were no sequence data.

Since human *Semaphorin 5* had been subcloned in pCDNA3.1/zeo™, which confers zeocin™ resistance to the transfected cells, a zeocin™ sensitivity assay was performed for NCI-H209 cells to determine the lowest zeocin™ concentration to be used for selecting stable transfectants. A zeocin™ concentration of 250 µg/ml was found to be sufficient to eliminate ~90% of untransfected NCI-H209 cells in 6 days.

This research has generated the necessary reagents for a future study looking at tumor suppression ability of human Semaphorin 5 in NCI-H209 cells. The gene for this protein has been subcloned into a suitable mammalian expression vector fused in-frame with a small tag, to allow detection by the use of antibodies. A zeocin™ sensitivity assay of NCI-H209 cells has been performed to determine what zeocin™ concentration to use for selecting stable transfectants. Furthermore, 5 assays have been outlined and briefly discussed, which can be done to test different tumor-like characteristics and ultimately determine if human Semaphorin 5 is indeed a suppressor of small cell lung carcinoma.

Chapter 2

Subcloning of Human *Semaphorin 5* (HS5)

[2.1] Original human Semaphorin 5 Bluescript clone (work done by Dr. John Minna's laboratory at University of Texas Southwestern Medical Center)

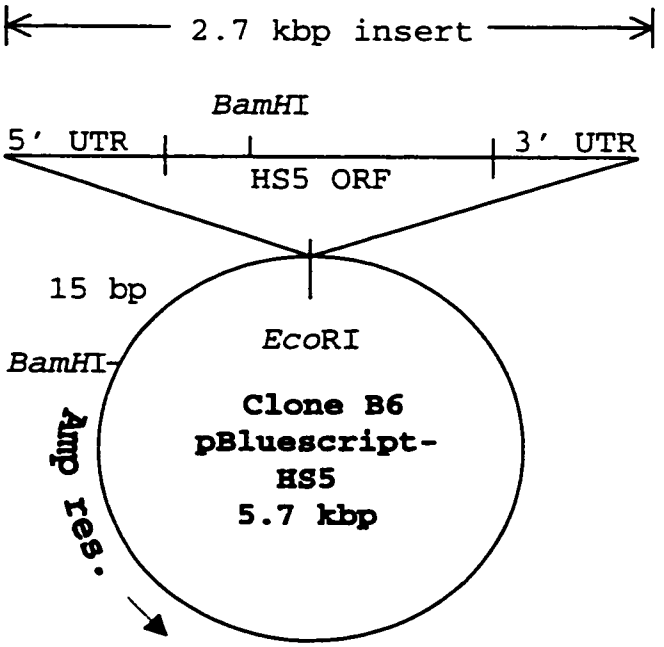
A human pancreatic islet cDNA library was screened with a contig covering 600 kbp localized to the 3p21.3 SCLC homozygous deletion region. Clone B6 was found to encode a *Semaphorin* related sequence of 2919 base pairs (Appendix 1), which was named human *Semaphorin 5* (GenBank Accession number U28369) (Sekido et al. 1996). The HS5 insert had been cloned into the *Eco*RI site of pBluescript vector (Stratagene, La Jolla, CA) (Fig. 1). The insert of clone B6 contains the entire open reading frame (ORF) of HS5, as indicated in Appendix 1, and is 2719 base pairs long, starting at nucleotide 197 of U28369.

[2.2] Verification of HS5 clone B6

Clone B6 of HS5 was generously provided by Dr. John Minna at University of Texas Southwestern Medical Center. Several diagnostic restriction digests were performed on the clone before beginning the construction of the expression construct. 1 µg of plasmid DNA from clone B6

was used to transform 40 µl of electrocompetent TOP 10F™ *E.coli* cells (Stratagene, La Jolla, CA). Electroporation was carried out using a BTX electroporator (model BT 720, BTX, San Diego, CA). A pulse of 2.0 kV was applied to the mixture of cells and DNA in a 0.5 cm gap electroporation cuvette (BTX, San Diego, CA). Immediately after the pulse, 1 ml of 37°C SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose) was added to the electroporated cells. The cells were then incubated at 37°C for one hour with gentle shaking (~250 rpm). The entire transformation mixture (~ 1.04 ml) was then spread on LB-agar plates containing 50 µg/ml ampicillin (the vector pBluescript confers ampicillin resistance as indicated in Fig. 1).

Fig. 1
Schematic representation of clone B6 containing HS5 in pBluescript



Plasmid DNA was isolated from two of the resulting colonies by the alkaline lysis mini preparation protocol (Ausubel et al. 1997, 1-16). The plasmids from both colonies were digested with *Eco*RI and the digests were run on a 1% agarose gel in TBE buffer containing 1 µg/ml ethidium bromide (Sigma, St. Louis, MO). As expected, two fragments resulted from the digestion of each clone: the 2.7 kbp insert and the 3 kbp pBluescript vector (data not shown).

A second diagnostic restriction digest was performed with *Eco*RI and *BamHI*. As indicated in Appendices 1 and 2,

as well as Fig. 1, there is a *BamHI* site in HS5 ORF. A *BamHI* site is also present in pBluescript vector, 15 bases upstream of the *Eco*RI cloning site (Fig. 1). Again the expected fragments resulted from this double digest: ~740 bp, 2 kbp, and the 3 kbp pBluescript vector (data not shown). Based on these results, clone B6 appeared to contain the correct HS5 insert (U28369).

[2.3] Amplification of HS5 Open Reading Frame

To generate an expression construct containing the smallest HS5 insert (with the complete ORF), thus eliminating any uncharacterized potential regulatory sequences in the 5′ and/or 3′ untranslated regions, primers were designed to amplify HS5 ORF only (~2.2 kbp, see Appendix 1) using PCR. The forward primer (Table 1) included the 6 nucleotide Kozak sequence preceding the first methionine. This short sequence is thought to modulate translation by eukaryotic ribosomes (Kozak 1986).

The reverse primer (Table 1) was designed to omit the stop codon, to allow for the construction of an in-frame HS5-tagged fusion protein. As there are currently no antibodies available against HS5, the inclusion of a tag with readily available antibodies to it would allow

detection of the fusion protein, and confirmation of HS5 expression. The tag chosen for the HS5 expression construct was the commonly used myc epitope (Evan et al. 1986).

The primers were designed for use with *Pfu* DNA polymerase (Stratagene, La Jolla, CA), a high fidelity polymerase possessing a 3′ to 5′ exonuclease activity. To prevent primer degradation, a single phosphorothioate bond was incorporated at the 3′ end of each primer, replacing a phosphodiester bond (Skerra 1992).

Table 1
PCR primers for amplification of HS5 ORF from clone B6 (pBluescript-HS5)

Primer	**Sequence**
HS5 Forward	5′-GCTGAGATGGGGCGGGCCGGGGC*T-3′
HS5 Reverse	5′-CCAGTGCGTTGCGCTGCGCG*G-3′

The asterisk represents the presence of a phosphorothioate bond at the 3′ termini. Primers were purchased from Operon Technologies (Santa Clara, CA).

The primers were designed to have similar GC content of ~75%. Predicted melting temperatures of 77°C and 72°C for HS5F and HS5R, respectively, were calculated using the

following formula: $T_m(°C) = 2(N_A+N_T)+4(N_G+N_C)$. A 100 µl PCR run was set up according to the *Pfu* DNA polymerase supplier's protocol (Stratagene, La Jolla, CA) and carried out using a thermal controller (PTC-100, MJ Research Inc., MA) with the following conditions: T_{melt}=95°C for 45 seconds, T_{anneal}=69°C for 45 seconds, and T_{extend}=72°C for 5 minutes. The reaction was run for 17 cycles followed by a 10 minute extension at 72°C. Though typical reactions have 25-30 cycles, it was found that the product yield was high enough with only 17 cycles of amplification. Therefore, to reduce the possibility of accumulated errors, the amplification for subsequent cloning of HS5 was performed with 17 cycles.

[2.4] Gel purification of the amplified product (HS5 ORF)

The DNA in the 100 µl PCR was precipitated with ethanol and loaded in its entirety onto a 1% agarose gel containing 1 µg/ml ethidium bromide. The desired PCR product (2.2 kbp) was cut out of the gel and purified by binding to a silica matrix (Gene Clean Kit, Bio 101, La Jolla, CA). During the manipulation of the gel, the exposure to UV light was kept at a minimum to prevent any possible damage to DNA.

[2.5] Linearization and dephosphorylation of mammalian expression vector pCDNA3.1/zeo™

The ORF of HS5 was to be subcloned into the mammalian expression vector pCDNA3.1/zeo™ (Invitrogen, San Diego, CA). Since *Pfu* DNA polymerase has 3' to 5' exonuclease activity, almost all of the PCR products produced are expected to have blunt ends. For this reason, the vector pCDNA3.1/zeo™ needed to be restricted with an enzyme that would also generate blunt ends, so that it could be ligated to the blunt ended insert.

*Eco*RV was used to cut within the multiple cloning site and linearize pCDNA3.1/zeo™. The restriction enzyme was then inactivated at 65°C for 25 minutes. Following the heat inactivation, a phenol/chloroform extraction was performed and the DNA was precipitated with ethanol. The linearized vector was then treated with calf intestinal alkaline phosphatase (Pharmacia Biotech, Piscataway, NJ) to remove the 5' phosphates and decrease the possibility of vector reannealing during the subsequent ligation. The phosphatase was inactivated by heating to 70°C for 10 minutes.

[2.6] Ligation, bacterial transformation, and plasmid DNA preparation

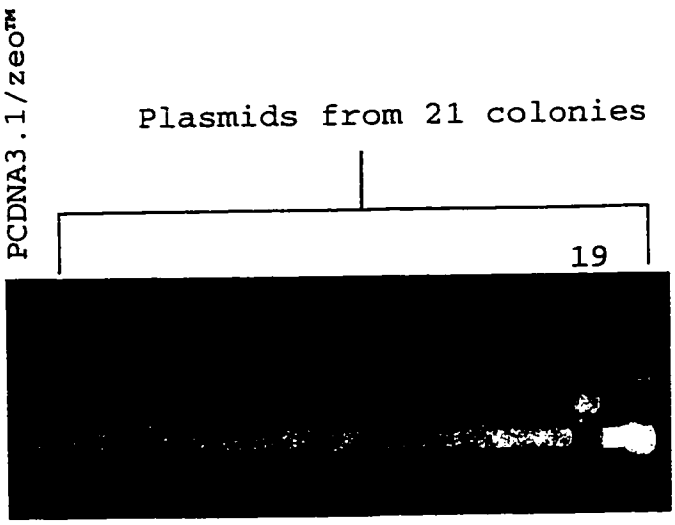
The gel-purified insert was polished by filling in any incomplete 3' ends with DNA polymerase and phosphorylating 5' ends with polynucleotide kinase to generate blunt ended products with 5' phosphates (reagents and protocols obtained from Perfectly Blunt Cloning Kit, Novagen, Madison, WI). Following this pre-treatment, a ligation was carried out using Novagen's reagents and protocol.

The ligation mixture was used to transform electrocompetent TOP 10F™ *E.coli* cells as before. Electroporated cells were then spread on LB-agar plates containing 50 µg/ml ampicillin. In addition to zeocin™ resistance, which is used to select for transfected mammalian cells, the vector pCDNA3.1/zeo™ also confers ampicillin resistance.

Twenty one colonies were obtained. Possible reasons for this low ligation efficiency could include the thorough dephosphorylation of the linearized vector, to significantly reduce reannealing, as well as the inherent low efficiency of a blunt ended ligation. Plasmids were isolated from all 21 colonies by the alkaline lysis mini preparation protocol as before. The uncut plasmids were

run on a gel to identify clones that had taken up the 2.2 kbp insert. As a control, the uncut vector was run along with the 21 samples (Fig. 2).

Fig. 2
Uncut plasmids of 21 colonies resulting from transformation



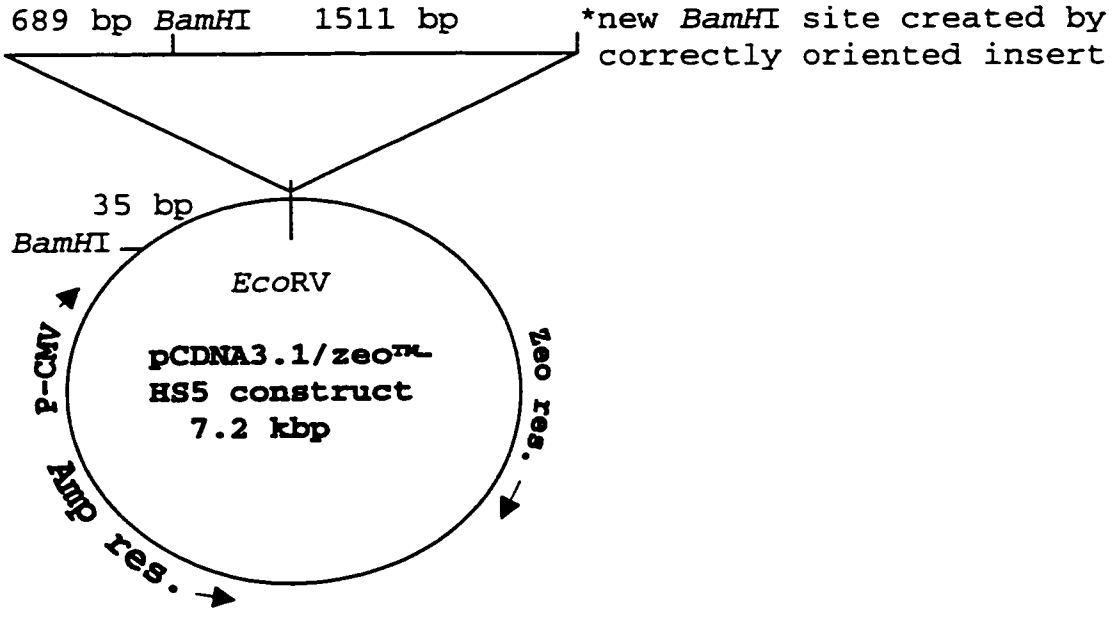
Lane 1 is pCDNA3.1/zeo™ control.

Based on the gel shown in Fig. 2, clone #19 alone appeared to have taken up the 2.2 kbp insert. Note that clone #19 migrated slower than the other clones. The other 20 clones migrated in an identical manner as pCDNA3.1/zeo™ vector.

[2.7] Insert orientation

Since both the insert and the linearized vector had blunt ends, it was equally possible for the insert to be ligated in either the forward or the reverse orientation. A *BamHI* digest of clone 19 would reveal the orientation of the insert. *BamHI* will cut the 2.2 kbp HS5 ORF insert asymmetrically (Appendix 1 and Fig. 3). As indicated in Fig. 3, there is also a *BamHI* site in the multiple cloning site of the vector (between the promoter and the insert), 35 bases upstream of the original *Eco*RV cloning site. If the insert were ligated in the correct orientation, as shown in Fig. 3, a third *BamHI* site would be created at the ligation junction.

Fig. 3
A representation of pCDNA3.1/zeo™-HS5 construct created by ligation of the 2.2 kbp HS5 ORF into the *Eco*RV site of pCDNA3.1/zeo™

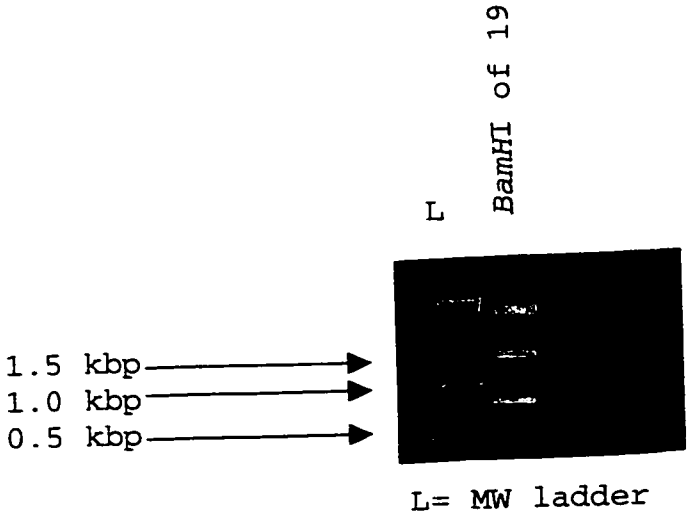


P-CMV = cytomegalovirus promoter

Clone 19 was digested with *BamHI* to determine the insert orientation. Note that if the insert were ligated in the indicated orientation (Fig. 3), there would be three restriction fragments created by a *BamHI* digest: 724 bp, 1.5 kbp, and 5 kbp. Note also the new *BamHI* site created if the insert were in this orientation. If the insert were ligated in the reverse orientation, there would be no new *BamHI* sites created at either end, and a *BamHI* digest would

yield two fragments: 1.5 kbp and 5.7 kbp. Fig. 4 shows the results of this digest.

Fig. 4
Results of the *BamHI* digest of clone 19



As shown in Fig. 4, the *BamHI* digest of clone 19 generated three fragments: 724 bp, 1.5 kbp, and 5 kbp. Based on these results, clone 19 appeared to have the 2.2 kbp HS5 ORF insert in the desired orientation.

[2.8] Insertion of the myc epitope

The next part of cloning involved the insertion of a sequence encoding a small peptide, the myc epitope, in-frame with HS5. The myc epitope is 10 amino acids long and can be used to detect the expression of HS5 since

antibodies are readily available to this small epitope. Note that the HS5 ORF insert was designed not to have a stop codon. The myc fragment would be inserted in-frame at the *Not*I/*Xba*I sites of the multiple cloning site of pCDNA3.1/zeo™ (Fig. 5). *Not*I and *Xba*I enzymes were chosen because there are no *Not*I or *Xba*I sites in the HS5 insert. The myc epitope was designed as two separate and complementary oligonucleotides (Table 2), with *Not*I and *Xba*I sites integrated.

It was decided to include the DNA sequence for the last 6 amino acids of HS5 (excluding the stop codon) in the design of the myc oligonucleotides, to partially restore the carboxy terminus of HS5, which might be important for function. The myc epitope insert is therefore referred to as myc+6.

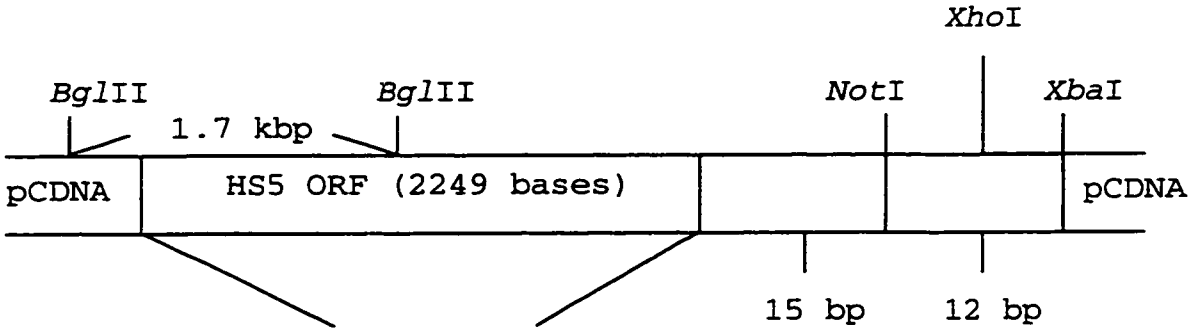
Table 2
Design of the myc+6 oligonucleotides

Myc+6F	5'-GGCCGC<u>GAGCAGAAGCTGATCTCCGAGGAGGACCTG</u>*CGCAGCGCAACG*
	*CACTGG*TGAT-3'
Myc+6R	3'- CG<u>CTCGTCTTCGACTAGAGGCTCCTCCTGGAC</u>*GCGTCGCGTTGC*
	*GTGACC*ACTAGATC-5'

<u>Underlined:</u> the actual DNA sequence of the myc epitope.
Italics: the DNA sequence of the last 6 amino acids of HS5, which were included in the design of these oligonucleotides (purchased from Operon Technologies).

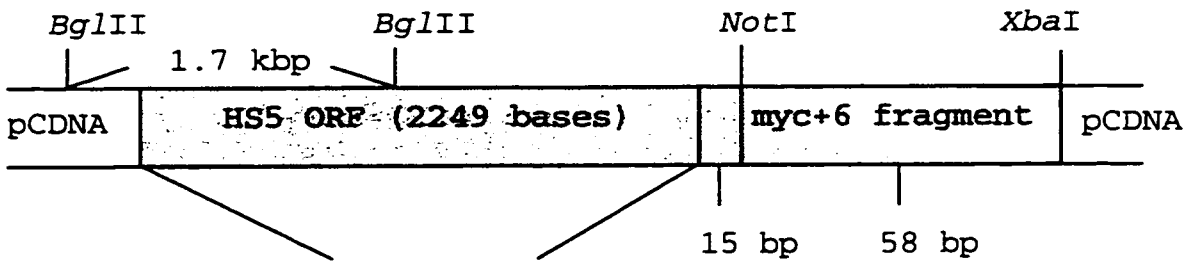
An equal concentration of each of the two myc+6 oligonucleotides (forward and reverse) were mixed together. To prevent non-specific annealing of the fragments, the mixture was heated to 95°C for 10 minutes and then allowed to cool to room temperature. The double stranded 58 base pair long fragment was now ready to be inserted into the *Not*I/*Xba*I sites of clone 19. Fig. 5A is a line representation of the construct without the myc+6 insert (clone 19). Fig. 5B is a representation of pCDNA3.1/zeo™-HS5-myc+6 construct.

Fig. 5A
A Line representation of pCDNA3.1/zeo™-HS5 construct (clone 19)



Former *Eco*RV cloning Site

Fig. 5B
A line representation of pCDNA3.1/zeo™-HS5-myc+6 construct



Former *Eco*RV cloning Site

The shaded area represents the HS5-myc+6 fusion protein.

Clone 19 was digested with *Not*I and *Xba*I. As indicated in Fig. 5A, this double digest would create a 12 base long fragment. After the digestion, the DNA was extracted with phenol/chloroform and precipitated with ethanol for only 30 minutes at -70°C so the 12 base long fragment in the supernatant would be selectively lost. The myc+6 fragment was then ligated into the *Not*I/*Xba*I cut pCDNA3.1/zeo™-HS5 clone 19 (reagents and protocols obtained from Novagen's Perfectly Blunt Cloning Kit).

After the ligation, an *Xho*I digest was performed on the ligation mixture to linearize all those plasmids that had not taken up the myc+6 insert (see Fig. 5). Linear plasmids do not transform well with the following

transformation protocol, hence pre-digestion with *Xho*I would reduce the number of false positives. Following the *Xho*I restriction, the mixture was used to carry out the following bacterial transformation (Chung and Miller, 1988).

Top 10F *E.coli* (Stratagene, La Jolla, CA) cells were grown to the early log phase (OD_{600}=0.3-0.6) in LB broth. The cells were then pelleted by centrifugation at 1000xg for 10 minutes at 4°C. Following centrifugation, the cells were resuspended in 1/10 volume of transformation and storage buffer (TSB): LB broth containing 10% polyethylene glycol (MW=3350), 5% DMSO, 10 mM $MgCl_2$, and 10 mM $MgSO_4$. The resuspended cells were incubated on ice for 10 minutes before being frozen at -70°C. For transformation, 100 µl of cells were pipetted into a cold polypropylene tube and mixed with 5 µl ligation/*Xho*I digest mixture. The tube was then incubated on ice for 25 minutes. Following this incubation on ice, 0.9 ml of TSB, containing 20 mM glucose, was added to the cells and the tube was incubated at 37°C for 1 hour with gentle shaking. The bacterial cells were then plated on LB-agar plates containing ampicillin.

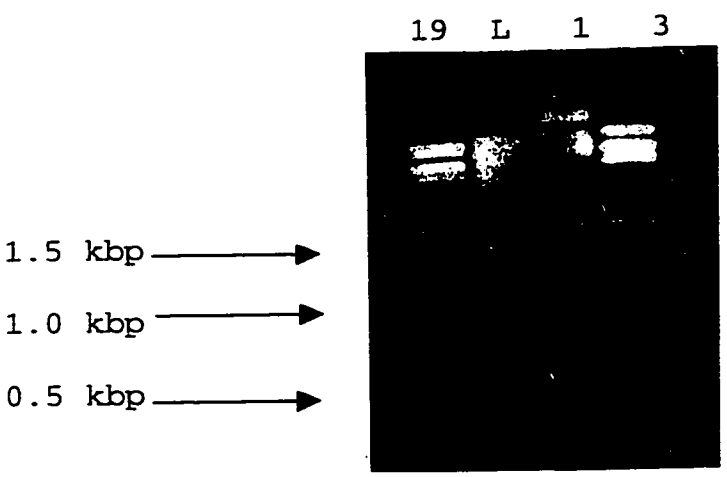
[2.9] Screening bacterial colonies for pCDNA3.1/zeo™-HS5-myc+6 construct

Plating the entire 1 ml transformation mixture yielded approximately 1000 colonies. Ten of the colonies were randomly selected for plasmid preparation. As indicated in Fig. 5, *Xho*I would linearize those plasmids that had *not* taken up the myc+6 insert. If, however, the myc+6 fragment had been successfully ligated, the *Xho*I site would be lost and the enzyme would not linearize the construct. The ten isolated plasmids were digested with *Xho*I and run on a 1% agarose gel along with uncut plasmids (data not shown).

Clones 1 and 3 appeared to have taken up the myc+6 insert since the *Xho*I cut plasmids and the uncut plasmids migrated in an identical manner on the gel, thus suggesting the loss of the *Xho*I site (data not shown). As a second confirmatory analysis, plasmids from these two clones were restricted with *Bgl*II and *Xho*I. As shown in Fig. 5, if the *Xho*I site were still present in between *Not*I and *Xba*I (i.e. no uptake of myc+6 insert), then this double digest would create three fragments: 1.7 kbp, 1.5 kbp (from *Bgl*II site of HS5 ORF to *Xho*I site of vector between *Not*I and *Xba*I), and 4 kbp. If however, the *Xho*I site were not present, indicating that the myc+6 insert had been successfully

ligated in, then the 1.7 kbp fragment would still be generated, but there would be no 1.5 kbp fragment because of the missing *Xho*I site. As a control, the original clone 19, which does have the *Xho*I site, was also digested with *Bgl*II and *Xho*I (Fig. 6).

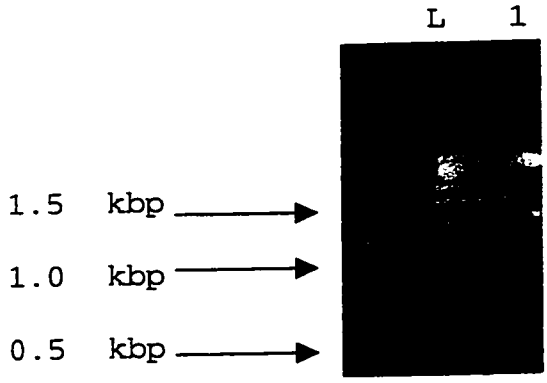
Fig. 6
*Bgl*II/*Xho*I double digest of the original pCDNA3.1/zeo™-HS5 clone 19 (lane 1), and clones 1 and 3, resulting from the transformation of clone 19 and myc+6 fragment ligation mixture (lanes 3 and 4, respectively)



The 1.5 kbp band was missing from the double digest of both clones 1 and 3 (Fig. 6), suggesting the loss of the *Xho*I site and the presence of myc+6 fragment. This band was, however, present on the double digest of clone 19, indicating the presence of the *Xho*I site, as expected.

Both clones 1 and 3 appeared to be the complete pCDNA3.1/zeo™-HS5-myc+6 construct. As one final check, the two clones were cut with *BamHI* to confirm the presence of the new *BamHI* site created by the ligation of HS5 ORF (Fig. 7). The digest of clone 3 (data not shown) did not yield the expected 1.5 kbp band, suggesting that the new *BamHI* site created by ligation of HS5 ORF was missing, throwing into question the frame continuity of HS5-myc fusion protein.

Fig. 7
BamHI digest of clone 1 (pCDNA3.1/zeo™-HS5-myc+6 construct)



Based on the above gel results, clone 1 appeared to still have the third *BamHI* site intact. Clone 1 was therefore considered to be the final expression construct, containing HS5 and myc+6 in-frame. Ultra pure plasmid DNA was isolated using Qiagen's plasmid preparation kit.

[2.10] Sequencing primers design and confirmatory PCR

Six sets of forward and reverse primers were designed (Table 3) and purchased from Operon Technologies. The annealing sites of each primer set (1F/1R; 2F/2R; etc.), except 6F/6R, were designed to be approximately 500 bases apart (as recommended by the sequencing facility). As indicated on the sequence in Appendix 2, annealing sites for primer set 6F/6R are approximately 200 bases apart.

Table 3
6 sets of Forward and Reverse sequencing primers

Primer	**Sequence**
1F	5' GTCTCCACCCCATTGACG 3'
1R	5' CAAACAGGCGTCCACGCT 3'
2F	5' ACGCCTGTTTGTGGGTGC 3'
2R	5' TTCTCGCTCTCCGGGATC 3'
3F	5' GAGAGCGAGAACCCAGAC 3'
3R	5' CCGCGCAAACTGGATGAC 3'
4F	5' CCAGTTTGCGCGGAACCA 3'
4R	5' AGCACAACGTGCTGGGGT 3'
5F	5' CGTTGTGCTCCGGAGACT 3'
5R	5' TTACGGCCCTTTCTCCGC 3'
6F	5' AAAGGGCCGTAACCGGAG 3'
6R	5' ACAACAGATGGCTGGCAACTAGAA 3'

Before sequencing the final construct, a PCR was performed with each set of primers. The results of this PCR would confirm the correct design of the primers, as

well as the correctness of the construct. The PCR was performed with *TaqPlus*™ DNA polymerase (Stratagene, La Jolla, CA) using the thermal controller as before. 10 µl of each reaction were loaded on a 1% agarose gel (Fig. 8).

Fig. 8A
PCR results using sequencing primer sets 1-5 with pCDNA3.1/zeo™-HS5-myc+6 expression construct as the template

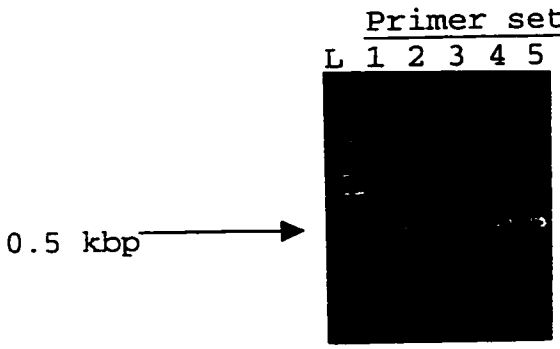
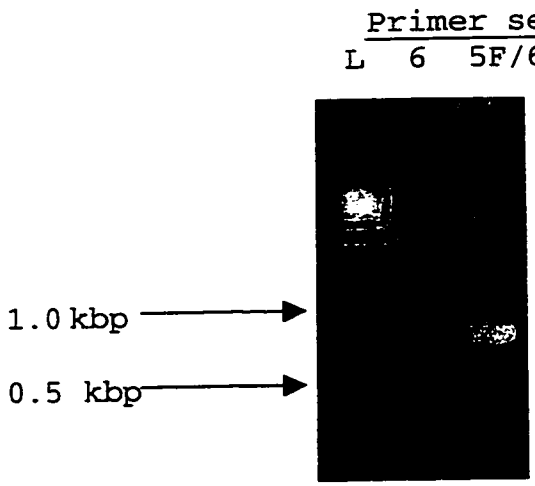


Fig. 8B
PCR results of sequencing primer sets 6 (lane 2) and 5F/6R (lane 3)



There was a 500 bp fragment in all 5 reactions (Fig. 8A). The expected ~200 base pair fragment, by using primer set 6, was also present (Fig. 8B). Primers 5F and 6R were used in another reaction as another confirmatory control (Fig. 8B). The expected PCR product (~500bp+~200bp = ~700bp) was clearly present in that reaction as well.

The construct was submitted to California State University Northridge for sequencing. Both forward and reverse strands were sequenced. The sequence data was compared to the published sequence of HS5 (U28369) and found to be identical with the exception of two bases, for which there were no sequence data. The sequence data is presented in Appendix 2.

Chapter 3

Transfection Trials of NCI-H209 Cell Line

[3.1] Characteristics of NCI-H209 cells

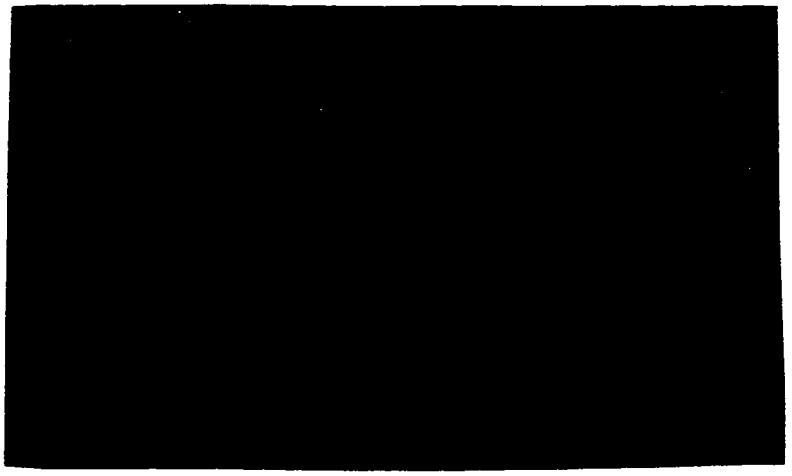
NCI-H209 cell line is a classic SCLC cell line, which was derived from the bone marrow of a Caucasian male with small cell cancer of the lung. This line expresses elevated levels of four biochemical markers: neuron-specific enolase, brain isozyme of creatine kinase, L-DOPA decarboxylase, and bombesin-like immunoreactivity (Carney et al. 1985). NCI-H209 cell line produces normal amounts of p53 mRNA relative to normal lung tissue (Hensel et al. 1990). There are no gross structural DNA abnormalities and the c-myc DNA sequences are not amplified (Little et al. 1983; Takahashi et al. 1989). These cells express an aberrant form of *RB1* that is not phosphorylated, possibly due to a single point mutation at codon 706, which changes a cysteine to a phenylalanine (Kaye et al. 1990). According to the depositors of this cell line (Carney et al. 1985) and American Type Culture Collection (ATCC), NCI-H209 cells grow as large aggregates in suspension (Fig. 1). No meaningful viability percentage can be measured for

these cells without damaging them by proteolytic treatment, as only the cells in aggregates are viable (Fig. 2).

Fig. 1
An aggregate of viable NCI-H209 cells (at 400x)

Note that this aggregate may contain ~200-1000 viable cells.

Fig. 2
NCI-H209 cells stained with trypan blue (at 10x)



Notice that only the clumped cells are viable.

[3.2] Culturing of NCI-H209 cells

The NCI-H209 cell line is missing the gene for Semaphorin 5 (Sekido et al. 1996), and therefore, is a suitable line for testing the tumor suppression ability of this Semaphorin. NCI-H209 cells were purchased from ATCC (Manassas, VA). The 1 ml ampule of frozen cells was thawed and the cells cultured in 10 ml of 37°C 90% RPMI 1640 (Gibco BRL, Rockville, MD) and 10% heat inactivated fetal calf serum. The culture medium also contained penicillin at 100 units/ml and streptomycin at 100 µg/ml. The culture was monitored daily and the medium renewed every 3 days by spinning down the cells, removing the old medium, and resuspending in fresh culture medium.

The growth rate was relatively slow. The number of viable cells increased by approximately 1 clump every 3 days (viable clumps were counted in the visual field of the inverted microscope at 200x). ATCC was contacted regarding the growth properties of this cell line. The response was that the growth rate of this line is significantly slower in the presence of antibiotics and the recommendation was to grow this cell line in an antibiotic free culture medium containing 20% non-inactivated fetal calf serum.

The cells were therefore re-cultured in 80% RPMI + 20% non-inactivated fetal calf serum with no antibiotics. Again the growth rate was monitored daily by visual inspection of the culture under an inverted microscope. The growth rate increased significantly (approximately 1 new clump per day). The color of the culture medium changed from red to slightly pinkish-yellowish, which is typical of a healthy growing culture, indicating a pH shift due to acid production during cell growth. This color change was never present in the previous culture medium. Also, as observed under the microscope, there were more viable clumps each day. The culture medium was renewed every 3 days.

[3.3] Initial zeocin™ sensitivity assay

In order to generate stable transfectants expressing HS5-myc, which had been cloned in pCDNA3.1/zeo™ (see Chapter 2), the lowest lethal zeocin™ concentration to eliminate untransfected cells had to be determined. Knowing the initial cell concentration was very crucial for starting this assay. After vigorous up and down pipetting of a growing culture, 1 ml was removed for counting. As stated earlier, viable NCI-H209 cells only grow as

irregularly shaped clumps. The purpose of pipetting the culture was to break these clumps into single cells and make counting easier. This procedure, however, did not achieve the goal of dissociating the clumps. As suggested by ATCC, it was assumed that each clump consisted of a single layer of cells. A very rough estimate of cell density was obtained (~500,000/ml).

Approximately 10^5 NCI-H209 cells were plated in each of the wells of a 6-well culture plate. Dilutions were made to have a volume of 4 ml in each well. Zeocin™ was added to each well at the following final concentrations: 0, 50, 250, 500, 750, and 1000 µg/ml. The culture media, with proper zeocin™ concentrations, were renewed every 3 days.

It continued to be difficult to obtain accurate counts of these cells and determine percent viability. After discussing the problem with ATCC, the recommendation was to simply look at the wells under an inverted microscope and determine whether viable clumps were present at the indicated zeocin™ concentrations. Following this procedure, it was found that there were still viable clumps in the 500 µg/ml zeocin™ well after 6 days. The lowest zeocin™ concentration to eliminate untransfected NCI-H209 cell in 6 days was therefore estimated to be ~750 µg/ml.

It was understood, however, that this determination was only a rough estimate and not one based on accurate cell counts and percent viability determination. The only means by which this decision was made, was the visual inspection for the presence of viable clumps of cells.

[3.4] Electroporation trial

The NCI-H209 cells have been successfully transfected by electroporation (Johnson et al. 1986). Since this electroporation had succeeded in generating stable NCI-H209 transfectants, it was decided to follow the referenced protocol (Potter et al. 1984) and carry out an electroporation experiment with the following modifications/specifications:

- Since electroporation requires the construct to be linear, the pCDNA3.1/zeo™-HS5-myc construct had to be linearized with a restriction enzyme that would cut only once in a non-essential region. *Mun*I was used to cut and linearize the pCDNA3.1/zeo™-HS5-myc construct.
- The BTX electroporator (model BT 720, BTX, San Diego, CA) was used to carry out the electroporation experiment. The electroporator used by Potter et al. was an ISCO model 494

with voltage and current settings of 2 kV and 0.9 mA, respectively. The BTX electroporator does not have an adjustable dial for the current. As recommended by the BTX manufacturer, the voltage was set to 1 kV for the following electroporation experiments.

Two electroporation experiments were done: one with the pCDNA3.1/zeo™-HS5-myc construct and one with the vector alone. Zeocin™ was added 72 hours post-transfection at 750 µg/ml. The culture medium was renewed every 3 days. The cultures were monitored daily under an inverted microscope. After day 5, no more viable clumps could be observed in either culture. All cells appeared dead under the microscope. The cultures were monitored for an additional week, but no new viable clumps appeared. This suggested that the electroporation had failed.

[3.5] Lipofectamine™ transfection

Another transfection trial was set up using the Lipofectamine™ reagent (Gibco BRL, Rockville, MD). According to Gibco BRL, this reagent is well suited for transfections of suspension cultures. Since NCI-H209 cells grow in suspension, it seemed reasonable to try this

reagent. The manufacturer's protocol was followed with the following specifics:

- 3 µg of DNA and 12 µl of Lipofectamine™ were used for each transfection.

Again two transfections were done: one with the HS5 construct and one with the vector only. Zeocin™ was added 72 hours post-transfection at 750 µg/ml. As before, the culture medium was renewed every 3 days. After two weeks of monitoring the cultures, no viable clumps were observed. It appeared that this transfection had failed as well. A possible reason for this failure may be that the very few cells, which might have been successfully transfected, were eliminated by the high zeocin™ concentration.

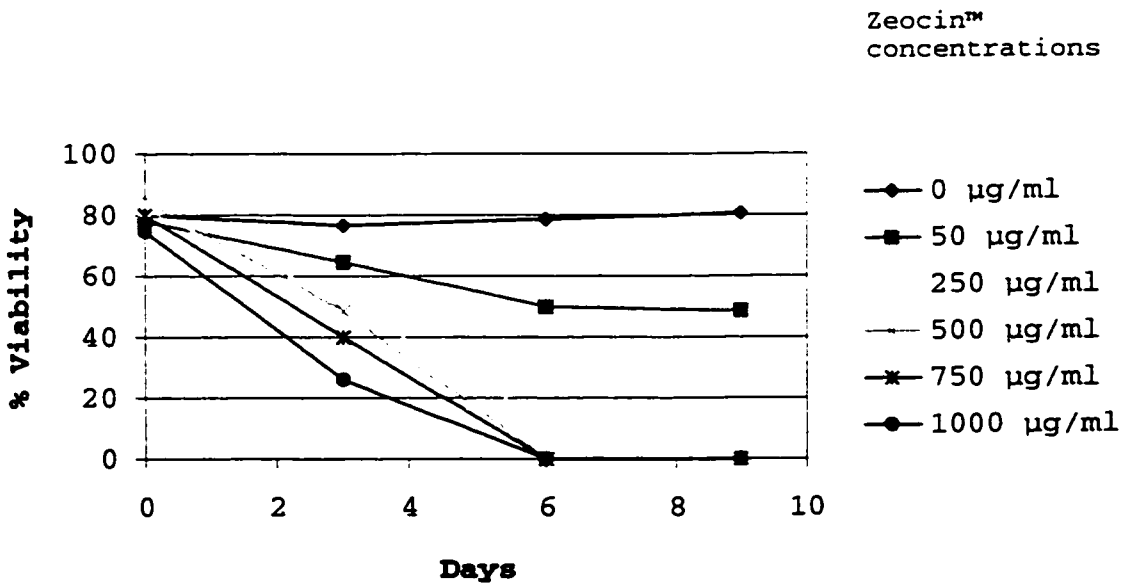
[3.6] Repeating the zeocin™ sensitivity assay

After the last two transfection failures, it was decided to repeat the zeocin™ sensitivity assay and generate a viability curve. After consulting with Dr. Harris Goonewardena (researcher at Dr. Matthes' laboratory, San Jose State University, Dept. Biological Sciences, San Jose, CA) and Dr. Steven White (Biology professor, San Jose State University) about the problem faced with obtaining

accurate counts with these clumped cells, it was decided to treat the cells with trypsin-EDTA before counting. A 5 ml aliquot of the culture was spun down to pellet the cells. The cells were washed once with PBS, and after centrifugation, they were resuspended in PBS containing 0.5% trypsin and 0.53 mM EDTA. The cells were then incubated at 37°C for 5 minutes. After 5 minutes, trypsin was inactivated by the addition of complete culture medium containing fetal calf serum. The cells were then counted. Most of the clumps had broken up into single cells. There were still some smaller clumps present, but they were small enough that individual cells could be counted within the clump.

The zeocin™ sensitivity assay was repeated. Approximately 10^6 cells were seeded in each of 6 T-25 culture flasks. Zeocin™ was added to the flasks at the previously indicated concentrations. Following the above trypsinization procedure, cells were counted on days 0, 3, 6, and 9. Percent viability was determined for each zeocin™ concentration on each counting day. A viability curve was generated for NCI-H209 cells (Fig. 3).

Fig. 3
Percent viability of NCI-H209 cells incubated with varying concentrations of zeocin™



As shown in Fig. 3, a zeocin™ concentration of 250 µg/ml would eliminate approximately 90% of untransfected NCI-H209 cells in 6 days. This zeocin™ concentration was significantly lower than that used in the previous two transfection trials (750 µg/ml), suggesting that one of the possible reasons no transfectants were obtained might have been the extremely high zeocin™ concentration. Being equipped with this new information, another transfection was attempted.

[3.7] Lipofectin™ transfection

Lipofectin™ (Gibco BRL, Rockville, MD) is another form of a liposome reagent that has been successfully used to transfect suspension cultures. It is a slightly modified form of Lipofectamine™, and according to Gibco, performs better with suspension cultures.

Three transfections were set up according to the supplier's protocol (3 µg DNA/transfection and 12 µl Lipofectin™): one with the pCDNA3.1/zeo™-HS5-myc construct, one with the vector alone, and one mock transfection with no DNA at all. Since transfection had to be carried out in serum-free culture medium, the purpose of the mock transfection was to see what effect serum starvation would have on these cells. Zeocin™ was added, only to the two DNA containing transfection mixtures, 72 hours post-transfection at 250 µg/ml. Cells were monitored daily and culture medium renewed every 3 days.

Viable clumps could be observed up until the fifth day post-transfection in both zeocin™ containing cultures. As indicated in Fig. 3, a significant number of untransfected cells would still be viable at this zeocin™ concentration after 5 days (~25%). After day 5 however, very few viable

clumps were found in the cultures, and by the 8th day, all cells appeared dead.

The fact that the mock-transfected cells survived indicated that the serum starvation, which is part of the procedure, was not the cause of the transfection failure. There are different parameters associated with the Lipofectin™ transfection such as amount of DNA to be used, amount of Lipofectin™ to be used, and duration of the incubation time. One needs to empirically determine the optimal transfection conditions suitable for each cell line.

Chapter 4

Conclusion

The main accomplishments of this master's thesis project have been 1) creating a mammalian expression construct of human *Semaphorin 5*, and 2) characterizing the *in vitro* growth properties of a classic SCLC cell line, NCI-H209. The open reading frame of HS5 (excluding the stop codon) was amplified from an original clone (B6), containing the complete coding sequence plus 5' and 3' untranslated regions. The amplified PCR product was cloned into the mammalian expression vector pCDNA3.1/zeo™. A DNA fragment encoding a small peptide of 10 amino acids, the myc epitope, was cloned in-frame with HS5, to allow detection of the fusion protein using anti-myc antibodies. The final pCDNA3.1/zeo™-HS5-myc construct was confirmed by several diagnostic restriction digests before it was sent to California State University Northridge for sequencing. The construct sequence was shown to be identical to the published sequence (GenBank U28369), with the exception of two bases, for which no sequence data were available (see Appendix 2).

Electroporation was chosen as the transfection method, since stable NCI-H209 transfectants have been generated using electroporation (Johnson et al. 1986). The transfection was carried out using the BTX electroporator. This electroporator is not recommended by the manufacturer for mammalian cells, mainly because the only control dial on the instrument is the voltage control. The previously referenced electroporation experiment with this cell line (Potter et al. 1984; Johnson et al. 1986) utilized a more elaborate instrument, ISCO model 494, with control dials for voltage, wattage, and current. These variables were adjusted accordingly in an attempt to identify electroporation conditions that worked for NCI-H209 cells (see Chapter 3).

It was understood that the BTX electroporator would not generate the optimal electroporation conditions. Nevertheless, the experiment was carried out with the idea being that if even a single cell takes up the construct, it will eventually multiply and result in a stable cell line.

In order to select for transfectants, zeocin™ was added 72 hours after electroporation, at a concentration of 750 µg/ml. As mentioned in Chapter 3, the determination of this zeocin™ concentration was not based on accurate cell

counts and percent viability calculations. After 5 days of monitoring the electroporated cultures and renewing the medium with proper zeocin™ concentration, it became obvious that the electroporation had failed. No viable cells were present under the microscope.

The most logical possible reason for this electroporation failure was the choice of electroporator. The electroporator used by Potter et al. (1984) had adjustable dials for current and wattage, which allowed for manipulation of these parameters in order to achieve the optimal electroporation settings. As mentioned earlier, the BTX electroporator does not have control dials for these parameters. It is therefore possible that as a result of the pulse, most or all of the cells died and those that did survive, and possibly took up the DNA, were eliminated after the addition of a relatively high concentration of zeocin™.

Another plausible reason, which is not restricted to electroporation, could be the morphology of this cell line. NCI-H209 cells grow as large, dense floating aggregates. In reality then, only the cells on periphery of these clumps have access to the DNA. This could tremendously

reduce the efficiency of transfection, which by itself is not a very efficient process.

A second transfection was attempted using the Lipofectamine™ reagent from Gibco. According to the manufacturer, this liposome reagent has performed well with suspension culture transfections. The construct does not need to be linearized with this procedure. The most important precautions to consider with this method are 1) antibiotic-free medium MUST be used throughout the transfection, and 2) the initial incubation MUST be done in serum-free medium.

As with electroporation, there are several parameters associated with this lipofection, which also need to be optimized for a successful transfection. How much DNA to use, how much Lipofectamine™ to use, how many cells to use per transfection (this is especially difficult with this particular cell line), and how long to incubate in the absence of serum are all crucial considerations involved in this method of transfection. The manufacturer supplies a protocol providing a range for all the above mentioned quantities (i.e. how much DNA, etc.). For our transfection trial, the average quantities were used.

The second zeocin™ sensitivity assay was a success. After discussing the problem of accurately counting these cells with Drs. Goonewardena and White (San Jose State University), it was decided to gently treat the cells with trypsin-EDTA before counting, in hope of breaking up the clumps. The procedure, as explained in detail in the previous chapter, did indeed result in obtaining accurate viable cell counts. It was found that a zeocin™ concentration of 250 µg/ml would be sufficient to eliminate ~90% of untransfected NCI-H209 cells in 6 days (see Figure 3 in Chapter 3).

A third transfection was tried with the Lipofectin™ reagent. This reagent is also from Gibco and is structurally similar to Lipofectamine™. Lipofectin™ has been used with suspension cultures more often and has generated stable transfectants (Kaye et al. 1990). The transfection protocol is identical to that of Lipofectamine™. In addition to two side by side transfections carried out thus far, both with the electroporation and the Lipofectamine™, (one with the HS5-myc construct and one with the pCDNA3.1/zeo™ alone), a mock transfection was performed with no DNA added. The purpose of this mock transfection was to determine whether the

initial serum starvation step would have any effect on the viability of the cells.

Viable clumps of the DNA-transfected cells could be seen under the microscope up until the fifth day post-transfection. After day 5 however, the number of viable clumps decreased every day. No viable cells were present by day 10. The transfected cultures were maintained for a period of two weeks. The fact that the mock-transfected cells (cultured with no zeocin™) survived the procedure indicates that serum starvation alone was not the factor responsible for the transfection failure of the two DNA-transfected samples.

Overall, this research has set the foundation for a more detailed future study examining the tumor suppression ability of human Semaphorin 5 in NCI-H209 cells. The gene for this tumor suppressor candidate has been subcloned into the mammalian expression vector pCDNA3.1/zeo™, fused in-frame with a small tag (myc epitope). The zeocin™ sensitivity assay has revealed that a zeocin™ concentration of 250 µg/ml is sufficient to eliminate ~90% of untransfected NCI-H209 cells 6 days after transfection. In addition, 5 characteristic assays have been outlined and briefly explained, which can readily be performed on this

cell line and ultimately determine if human Semaphorin 5 is indeed a suppressor of small cell lung carcinoma.

One can also test the hypothesis that HS4 and HS5 *together* are suppressors of SCLC. Since NCI-H209 cells are missing the genes for both of these proteins (Sekido et al. 1996), this hypothesis could very well be tested with this particular cell line. The HS4 ORF can be cloned into the vector pCDNA3.1/neo, thus allowing for the selection of double transfectants (those cells resistant to both G418 and zeocin™). The effective G418 concentration to select for stable NCI-H209 transfectants has already been determined (Johnson et al. 1986). The same cloning procedure can be followed as with HS5 (Chapter 2), with the exception being that instead of the myc epitope, another small peptide called Flag (Sekido et al. 1996) would be inserted in-frame.

Once the HS4-Flag expression construct becomes available, it will be possible to generate three groups of stable NCI-H209 transfectants: 1) cells expressing *Semaphorin 5*, 2) cells expressing *Semaphorin 4*, and 3) cells expressing both *Semaphorins 4* and *5*. All of the previously mentioned assays can be performed with each of these cell lines. Although *Semaphorin 5* is deleted more

frequently in SCLC cells (see Chapter 1), therefore suggesting that *it* is the potential suppressor, a detailed future study as outlined above will determine if the tumor suppression ability is dependant on the expression of both these *Semaphorins*. It is possible that Semaphorin 4 and 5, when secreted, form a heterodimer and only then recognize and bind to a membrane receptor on SCLC cells. This interaction might then lead to intracellular signals controlling growth rate and proliferation.

If human Semaphorin 4 and/or Semaphorin 5 are found to be suppressors of SCLC cells, further studies should be done to identify receptors for these proteins. Possible mechanisms of action could then be proposed and tested. And eventually, gene therapy approaches can be utilized to specifically target SCLC cells in humans with this form of lung cancer.

Appendix 1
Translated DNA sequence of HS5 clone B6 (GenBank U28369)

Clone B6 was supplied by Dr. John Minna at University of Texas Southwestern Medical Center and contains the 2719 base pair insert (nucleotides 197-2916), which is boldfaced in the following sequence. The entire ORF of HS5 (nucleotides 236-2485) is contained within the insert of clone B6. Annealing sites for are shown for PCR primers as forward and reverse arrows.

```
TCTGTGATTGTGGCCAGGCGGGGCACCCTCGGAGGGGAGGGTTCGGAAGTGGAATGCGACC  61
  L  *  L  W  P  G  G  A  P  S  E  G  R  V  R  K  W  N  A  T

CCCCAGCCTCTTTCCCCTAGGGGCTGTAATCTGATCCCTGGGGACTCCCCCCCTAGCCTC  121
 P  Q  P  L  S  P  R  G  C  N  L  I  P  G  D  S  P  P  S  L

CCGCCCTCGCCCTCACTGCTGACTCCTCTTCCAGATCCTGGGGCAGAGTCCAGGGCAGCT  181
 P  P  S  P  S  L  L  T  P  L  P  D  P  G  A  E  S  R  A  A

                                                  Start of HS5
CAAGGCTCCTCCACACACACACCCGCTGAACCCTGAGCACCCTGAGCTGCTGAGATGGGG  241
 Q  G  S  S  T  H  T  P  A  E  P  *  A  P  *  A  A  E  M  G

CGGGCCGGGGCTGCCGCCGTGATCCCGGGCCTGGCCCTGCTCTGGGCAGTGGGGCTGGGG  301
 R  A  G  A  A  A  V  I  P  G  L  A  L  L  W  A  V  G  L  G

AGTGCCGCCCCCAGCCCCCCACGCCTTCGGCTCTCCTTCCAAGAGCTCCAGGCCTGGCAT  361
 S  A  A  P  S  P  P  R  L  R  L  S  F  Q  E  L  Q  A  W  H

GGTCTCCAGACTTTCAGCCTGGAGCGAACCTGCTGCTACCAGGCCTTGCTGGTGGATGAG  421
 G  L  Q  T  F  S  L  E  R  T  C  C  Y  Q  A  L  L  V  D  E

GAGCGTGGACGCCTGTTTGTGGGTGCCGAGAACCATGTGGCCTCCCTCAACCTGGACAAC  481
 E  R  G  R  L  F  V  G  A  E  N  H  V  A  S  L  N  L  D  N

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AACTGGGCAGGGAAGGACATTGGTACTGAGTGCATGAACTTCGTGAAGTTGCTGCATGCC  601
 N  W  A  G  K  D  I  G  T  E  C  M  N  F  V  K  L  L  H  A

TACAACCGCACCCATTTGCTGGCCTGTGGCACGGGAGCCTTCCACCCAACCTGTGCCTTT  661
 Y  N  R  T  H  L  L  A  C  G  T  G  A  F  H  P  T  C  A  F

GTGGAAGTGGGCCACCGGGCAGAGGAGCCCGTCCTCCGGCTGGACCCAGGAAGGATAGAG  721
 V  E  V  G  H  R  A  E  E  P  V  L  R  L  D  P  G  R  I  E
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 D  G  K  G  K  S  P  Y  D  P  R  H  R  A  A  S  V  L  V  G

GAGGAGCTATACTCAGGGGTGGCAGCAGACCTCATGGGACGAGACTTTACCATCTTTCGC  841
 E  E  L  Y  S  G  V  A  A  D  L  M  G  R  D  F  T  I  F  R

AGCCTAGGGCAACGTCCAAGTCTCCGAACAGAGCCACACGACTCCCGCTGGCTCAATGAG  901
 S  L  G  Q  R  P  S  L  R  T  E  P  H  D  S  R  W  L  N  E

                        BamHI
CCCAAGTTTGTCAAGGTATTTTGGATCCCGGAGAGCGAGAACCCAGACGACGACAAAATC  961
 P  K  F  V  K  V  F  W  I  P  E  S  E  N  P  D  D  D  K  I

TACTTCTTCTTTCGTGAGACGGCGGTAGAGGCGGCGCCGGCACTGGGACGCCTGTCCGTG 1021
 Y  F  F  F  R  E  T  A  V  E  A  A  P  A  L  G  R  L  S  V

             BglII
TCCCGCGTTGGCCAGATCTGCCGGAACGACGTGGGCGGCCAGCGCAGCCTGGTCAACAAG 1081
 S  R  V  G  Q  I  C  R  N  D  V  G  G  Q  R  S  L  V  N  K

TGGACGACGTTCCTGAAGGCGCGGCTGGTGTGCTCGGTGCCCGGCGTCGAGGGCGACACC 1141
 W  T  T  F  L  K  A  R  L  V  C  S  V  P  G  V  E  G  D  T

CACTTCGATCAGCTCCAGGATGTGTTTCTGTTGTCCTCGCGGGACCACCGGACCCCGCTG 1201
 H  F  D  Q  L  Q  D  V  F  L  L  S  S  R  D  H  R  T  P  L

CTCTATGCCGTCTTCTCCACGTCCAGCAGCATCTTCCAGGGCTCTGCGGTGTGCGTGTAC 1261
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AGCATGAACGACGTGCGCCGGGCCTTCTTGGGACCCTTTGCACACAAGGAGGGGCCCATG 1321
 S  M  N  D  V  R  R  A  F  L  G  P  F  A  H  K  E  G  P  M

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 H  Q  W  V  S  Y  Q  G  R  V  P  Y  P  R  P  G  M  C  P  S

AAGACCTTTGGCACCTTCAGTTCCACCAAGGACTTCCCAGACGATGTCATCCAGTTTGCG 1441
 K  T  F  G  T  F  S  S  T  K  D  F  P  D  D  V  I  Q  F  A

CGGAACCACCCCCTCATGTACAACTCTGTCCTGCCCACTGGGGGGCGCCCTCTTTTCCTA 1501
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CAAGTTGGAGCCAATTACACCTTCACTCAAATTGCCGCGGACCGGGTTGCAGCCGCTGAC 1561
 Q  V  G  A  N  Y  T  F  T  Q  I  A  A  D  R  V  A  A  A  D

GGACACTATGACGTCCTCTTCATTGGCACAGACGTTGGCACGGTGCTGAAGGTGATCTCG 1621
 G  H  Y  D  V  L  F  I  G  T  D  V  G  T  V  L  K  V  I  S

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 V  P  K  G  S  R  P  S  A  E  G  L  L  L  E  E  L  H  V  F
```

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TGCACCGAATGCTGTCTGGCGCGTGACCCCTACTGCGCCTGGGACGGGGTCGCGTGCACG 1861
 C  T  E  C  C  L  A  R  D  P  Y  C  A  W  D  G  V  A  C  T

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 V  E  G  S  S  A  F  L  E  C  E  P  R  S  L  Q  A  R  V  E

TGGACTTTCCAGCGCGCAGGGGTGACAGCCCACACCCAGGTGCTGGCAGAGGAGCGCACC 2101
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CCGGGCCCCAAACTCTGGTACCGGGACTTTCTGCAGCTGGTGGAGCCGGGCGGAGGTGGC 2341
 P  G  P  K  L  W  Y  R  D  F  L  Q  L  V  E  P  G  G  G  G

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TCGCGGAGAAAGGGCCGTAACCGGAGGACCCACGCCCCTGAGCCTCGCGCTGAGCGGGGG 2461
 S  R  R  K  G  R  N  R  R  T  H  A  P  E  P  R  A  E  R  G

                                End of HS5
<------------------------------|
CCGCGCAGCGCAACGCACTGGTGACCAGACTGTCCCCACGCCGGGAACCAAGCAGGAGAC 2521
 P  R  S  A  T  H  W  *  P  D  C  P  H  A  G  N  Q  A  G  D

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```

```
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 G  L  I  Y  *  Q  D  N  P  *  M  *  Q  P  R  E  G  G  T  G

CGGGCGCAGGATTCAGCCGGAGGGAAGGGACGGGGAAGCCGAGCTCCAGAGCAACGACCA 2761
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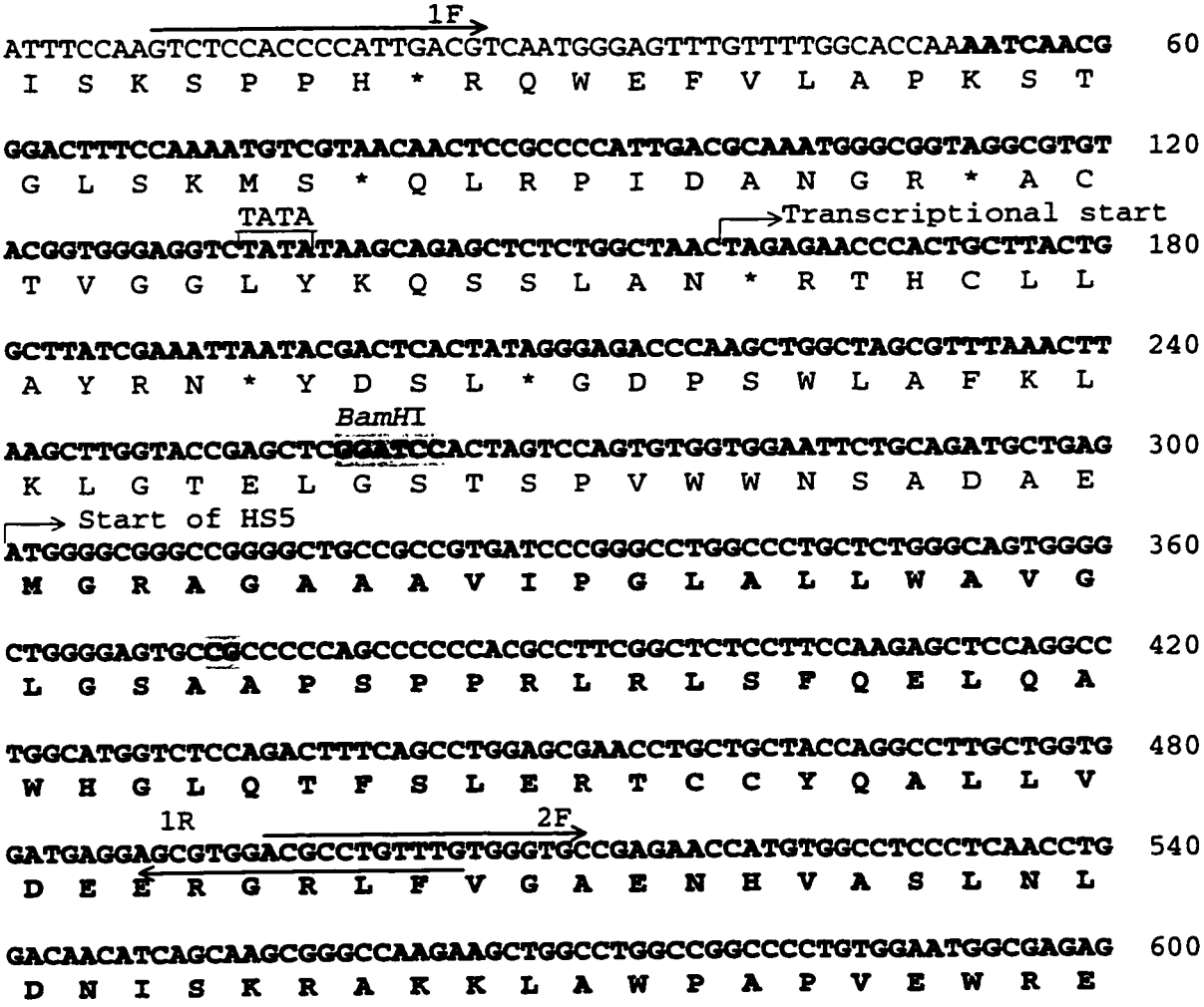
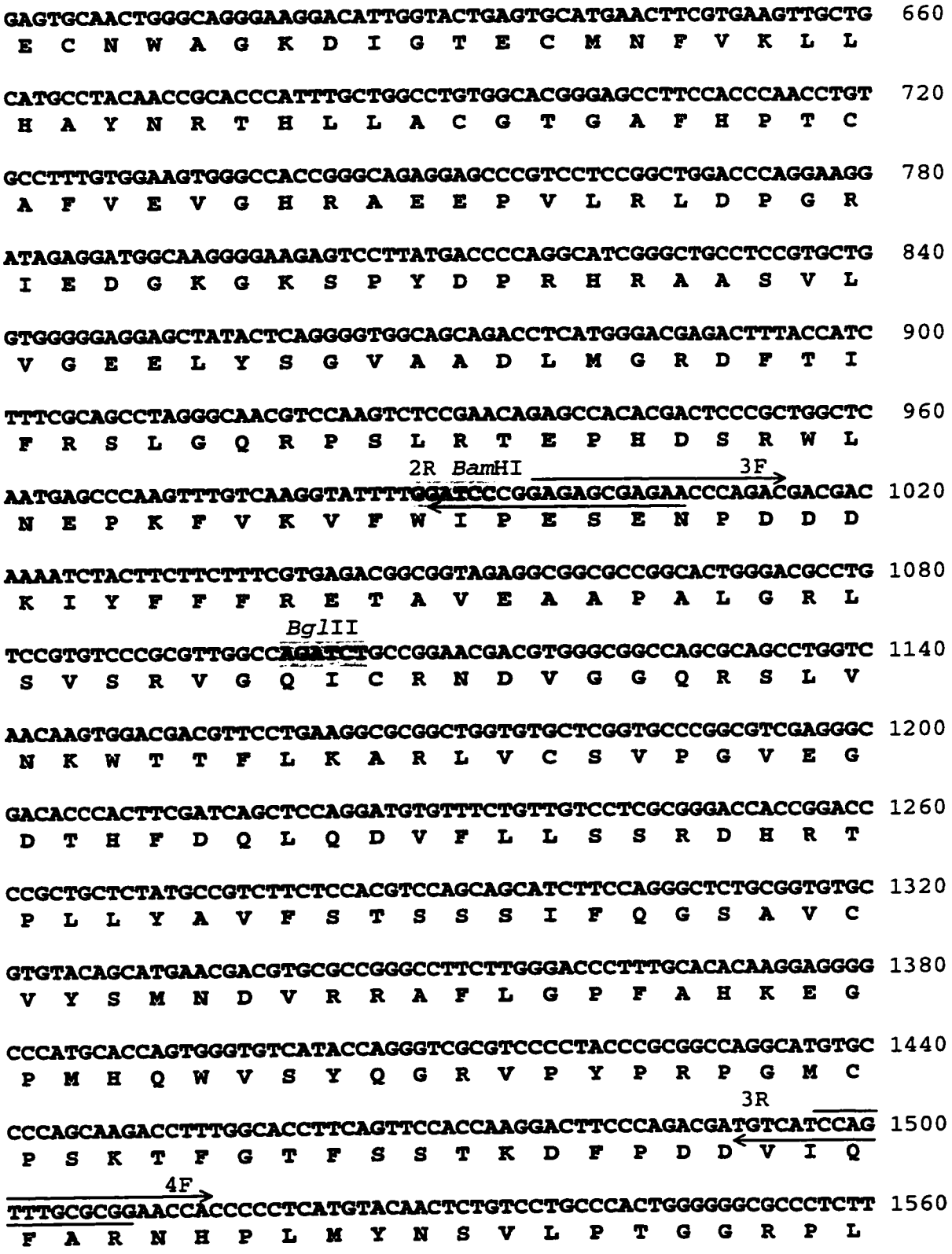
 E  Q  *  A  E  S  C  E  Q  A  G  L  L  E  V  G  R  G  R  P

ACTGTACTAAAGTAACGCAATAAACGCATTATCAGCCA                        2919
 T  V  L  K  *  R  N  K  R  I  I  S
```

Appendix 2
Nucleotide and amino acid sequence of pCDNA3.1/zeo™-HS5-myc+6 construct

Annealing sites are shown for each sequencing primer as forward or reverse arrows. The sequenced nucleotides as well as the amino acids of HS5-myc+6 fusion protein are boldfaced. Note the shaded GC at position 372 and 373. No sequence data for these 2 bases were obtained. Since there was no error in the entire sequenced region, it was therefore assumed that the sequence for these 2 bases was also correct.
BGH poly (A)=Bovine growth hormone polyadenylation signal

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H A Y N R T H L L A C G T G A F H P T C
GCCTTTGTGGAAGTGGGCCACCGGGCAGAGGAGCCCGTCCTCCGGCTGGACCCAGGAAGG 780
A F V E V G H R A E E P V L R L D P G R
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I E D G K G K S P Y D P R H R A A S V L
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V G E E L Y S G V A A D L M G R D F T I
TTTCGCAGCCTAGGGCAACGTCCAAGTCTCCGAACAGAGCCACACGACTCCCGCTGGCTC 960
F R S L G Q R P S L R T E P H D S R W L
2R BamHI 3F
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N E P K F V K V F W I P E S E N P D D D
AAAATCTACTTCTTCTTTCGTGAGACGGCGGTAGAGGCGGCGCCGGCACTGGGACGCCTG 1080
K I Y F F F R E T A V E A A P A L G R L
BglII
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S V S R V G Q I C R N D V G G Q R S L V
AACAAGTGGACGACGTTCCTGAAGGCGCGGCTGGTGTGCTCGGTGCCCGGCGTCGAGGGC 1200
N K W T T F L K A R L V C S V P G V E G
GACACCCACTTCGATCAGCTCCAGGATGTGTTTCTGTTGTCCTCGCGGGACCACCGGACC 1260
D T H F D Q L Q D V F L L S S R D H R T
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P L L Y A V F S T S S S I F Q G S A V C
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V Y S M N D V R R A F L G P F A H K E G
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P M H Q W V S Y Q G R V P Y P R P G M C
3R
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P S K T F G T F S S T K D F P D D V I Q
4F
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F A R N H P L M Y N S V L P T G G R P L

```
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 A  D  G  H  Y  D  V  L  F  I  G  T  D  V  G  T  V  L  K  V

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 I  S  V  P  K  G  S  R  P  S  A  E  G  L  L  L  E  E  L  H

GTGTTTGAGGACTCGGCCGCTGTCACCAGCATGCAAATTTCTTCCAAGAGGCACCAGCTG 1800
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 Y  V  A  S  R  S  A  V  A  Q  I  A  L  H  R  C  A  A  H  G

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```

4R (←) 5F (→)

```
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 V  E  W  T  F  Q  R  A  G  V  T  A  H  T  Q  V  L  A  E  E

CGCACCGAGCGCACCGCCCGGGGACTACTGCTGCGCAGGCTGCGGCGCCGGGACTCGGGC 2220
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GTGTACTTGTGCGCCGCCGTCGAGCAGGGCTTTACGCAACCGCTGCGTCGCCTGTCGCTG 2280
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GCGCCGCCGGGCCCCAAACTCTGGTACCGGGACTTTCTGCAGCTGGTGGAGCCGGGCGGA 2400
 A  P  P  G  P  K  L  W  Y  R  D  F  L  Q  L  V  E  P  G  G

GGTGGCAGCGCGAACTCCCTGCGCATGTGCCGCCCGCAGCCTGCGCTGCAGTCACTGCCC 2460
 G  G  S  A  N  S  L  R  M  C  R  P  Q  P  A  L  Q  S  L  P
```

5R (←) 6F (→)

```
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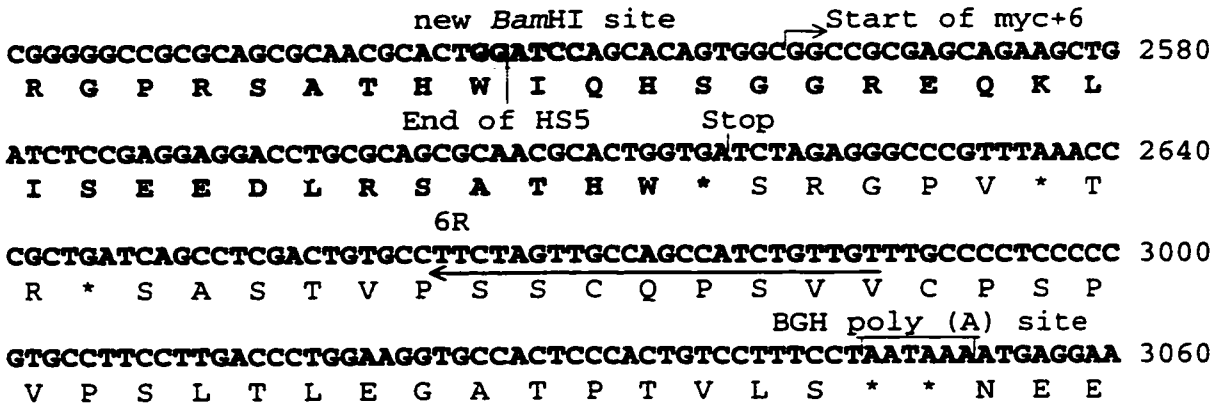
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End of HS5
Stop
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I S E E D L R S A T H W * S R G P V * T
6R
CGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCC 3000
R * S A S T V P S S C Q P S V V C P S P
BGH poly (A) site
GTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAA 3060
V P S L T L E G A T P T V L S * * N E E

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IMAGE EVALUATION
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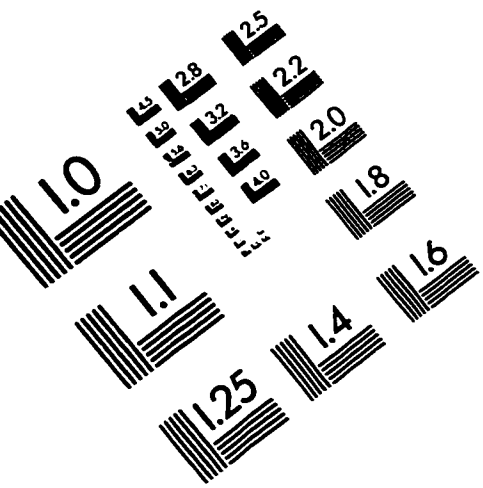

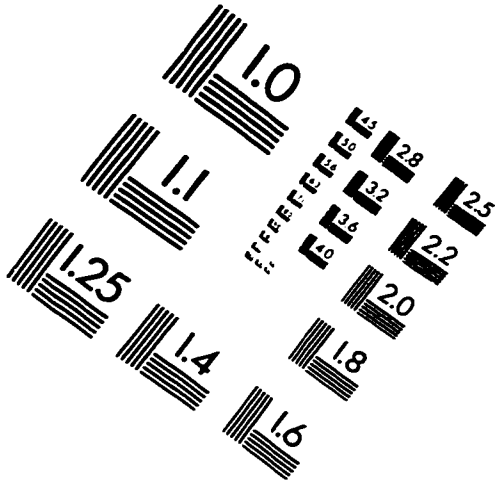

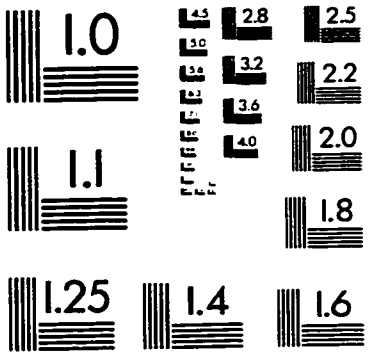

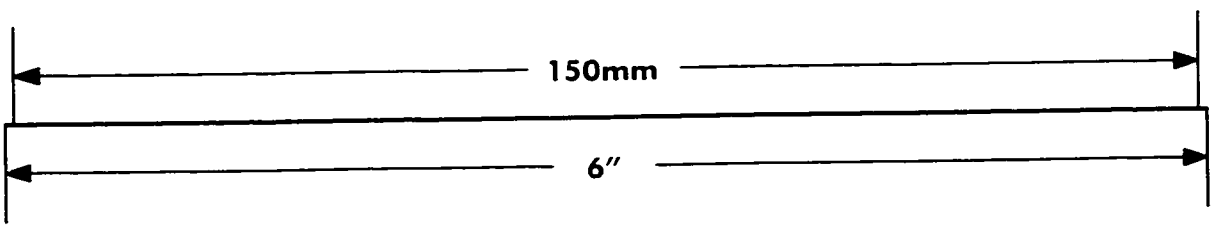

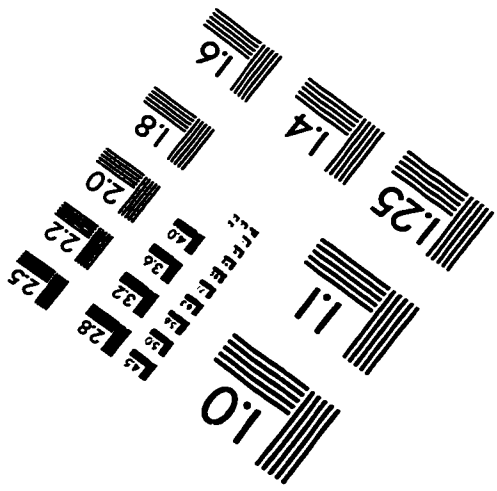

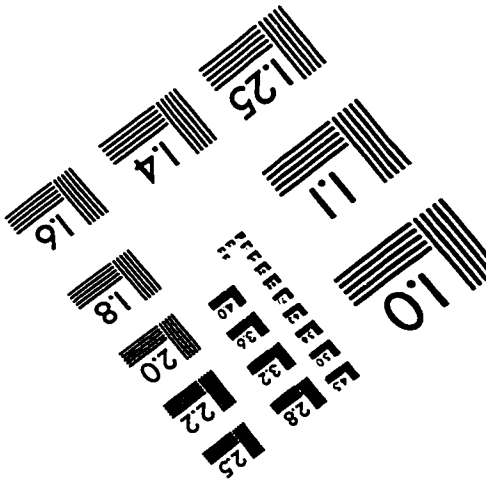

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